Preparation and Properties of Reduced κ-Casein

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κ-Casein obtained from pooled milk was resolved into three major and several minor components by polyacrylamide electrophoresis after reduction of the disulfide bonds with 2-mercaptoethanol. The reduced and alkylated derivatives quantitatively retained the ability to stabilize α_s-casein in the presence of calcium ions. Glycomacropeptide release and *para*-κ-casein formation were observed after the action of rennin on the alkylated κ-casein derivatives.

The electrophoretic behavior of κ-casein differs markedly from that of the other proteins of the casein complex. In starch-gel electrophoresis (1), κ-casein migrates as a broad unresolved zone, whereas the other casein proteins are resolved into distinct electrophoretic components. The electrophoretic behavior of κ-casein in polyacrylamide gels of low cyanogum content (5%) is similar to that observed in starch gels; however, in polyacrylamide gels of 7% cyanogum, the protein is almost totally retained in the sample slot (2). This behavior can be attributed to the greater molecular sieving effect exerted by polyacrylamide gels on the high molecular weight κ-casein aggregates.

The forces responsible for the formation of high molecular weight κ-casein aggregates are not well understood. However, it is unlikely that noncovalent forces are wholly responsible since the κ-casein complexes are only partially disrupted by dissociating solvents such as 5 *M* guanidine hydrochloride (3). On the other hand, the marked decrease in molecular weight of κ-casein brought about by alkaline pH's (4) or by reduction with 2-mercaptoethanol (3) indicates that intermolecular disulfide bonding probably contributes substantially to the formation of high molecular weight κ-casein.

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Reduction of the disulfide bonds of κ-casein has permitted the electrophoretic resolution of the reduced protein into a number of distinct components (5, 6). Neelin (5) has reported the resolution of κ-casein by starch-gel electrophoresis into two major *a* and *b* zones after reduction with either cysteine or mercaptoethanol. Similar results were obtained by Woychik (6) after polyacrylamide electrophoresis of reduced κ-casein. Both of these studies indicated qualitative and quantitative variation among the major components in κ-caseins from individual cows. Variations among the minor components were also reported in the latter study (6).

This communication elaborates on the previous studies and presents the results of further investigations of the effects of disulfide bond cleavage on the electrophoretic behavior and on some of the properties of κ-casein.

EXPERIMENTAL

Preparation of κ-casein. κ-Casein of pooled milk was prepared from acid-precipitated whole casein by the urea-sulfuric acid method of Zittle and Custer (7). The isolated κ-casein was further purified by precipitation of small amounts of other casein components from alcoholic solution according to the procedure of McKenzie and Wake (8). No trace of other casein components was found by polyacrylamide-gel electrophoresis of the purified κ-casein.

Reduction and alkylation of κ-casein. A 1–2%

solution of κ-casein was reduced for 1 hour in 8 *M* urea, pH 8.0, and veronal buffer (0.1 *M*) with 2-mercaptoethanol at a ratio of 10:1 based on a half-cystine content of 10 moles —SH per 100,000 gm protein (3). The reduced protein was then alkylated by the addition of a 3 *M* excess (based on total —SH content) of alkylating agent, and the pH was maintained at 8.0 by the addition of 0.1 *N* sodium hydroxide. After 15 minutes reaction with the alkylating agent, the pH was lowered to 5.0 and the solution was exhaustively dialyzed against distilled water to remove urea and salts. The protein precipitated during the dialysis and was redissolved by raising the pH of the solution to 8.0 by the addition of alkali, and the solution was then lyophilized. Cyanoethyl-κ-casein (CNE-κ-casein) was prepared by reaction with acrylonitrile (9); carboxymethyl-κ-casein (CM-κ) by reaction with iodoacetic acid; and carboxyamidomethyl-κ-casein (CAM-κ) by reaction with iodoacetamide.

A spectrophotometric method (10) utilizing potassium ferricyanide was used to check for the presence or absence of free sulfhydryl groups in the CNE-κ-casein. The completeness of reduction and alkylation of the liberated sulfhydryl groups in CNE-κ-casein was determined by amino acid analysis of the hydrolyzed protein. Carboxyethylcysteine, the acid hydrolysis product of cyanoethylcysteine, was eluted between serine and glutamic acid (9) according to the analytical system described by Piez and Morris (11).

α_s-Casein stabilization and rennin sensitivity. The ability of κ-casein and its reduced derivatives to stabilize α_s-casein against precipitation by calcium ions was measured according to the procedure described by Zittle (12). The tests were performed at pH 6.7 in the presence of 0.02 *M* calcium chloride with κ/α_s-casein ratios of 0.02/0.20. The results were expressed as percentage α_s-casein solubilized.

Rennin sensitivity was determined by measurement of the percentage total nitrogen soluble in 12% trichloroacetic acid (TCA) and as percentage total sialic acid soluble in 12% TCA. κ-Casein (0.5%) was digested with crystalline rennin (1 μg per milliliter) in pH 6.7 citrate (0.01 *M*) buffer containing 0.1 *M* sodium chloride. (Crystalline rennin was purchased from Sigma Chemical Company, St. Louis, Missouri.) Aliquots were removed during digestion and pipetted into equal volumes of 24% TCA. The precipitates were removed by filtration and the supernatants were analyzed for total nitrogen by the micro-Kjeldahl procedure, and for sialic acid by the method of Warren (13) after removal of TCA by dialysis.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis (PAE) was done in an E-C Apparatus Company[2] vertical electrophoresis cell. The gels were composed of 7% Cyanogum[3] in pH 9.2 tris-4.5 *M* urea buffer (14). Protein samples (20 μl of a 1% solution) were applied to the gel slots in pH 9.2 tris-7 *M* urea and run at 125 mA for approximately 3 hours. The electrophoretic patterns were developed by staining with Amido Black, and excess dye was removed with a 7% acetic acid wash.

When electrophoretic patterns were obtained of reduced nonalkylated κ-casein, the samples were prepared by the addition of 0.01 ml mercaptoethanol to 1.0 ml of a 1% solution in the tris-7 *M* urea buffer. Mercaptoethanol was initially incorporated into the gels (0.5 ml/150 ml gel solution) to prevent possible reoxidation of sulfhydryl groups during electrophoresis; however, subsequent studies showed this to be unnecessary.

RESULTS AND DISCUSSION

Reduction and electrophoresis of κ-casein. Whole casein can be resolved by polyacrylamide-gel electrophoresis into its component proteins, as illustrated in Fig. 1a. The principal casein components are readily resolved into discrete bands, whereas the presence of κ-casein in the electrophoretic pattern can be detected only as a sharp dark line at the leading edge of the sample slot. The pattern of κ-casein (Fig. 1b) more clearly illustrates its electrophoretic behavior in polyacrylamide gels, wherein, except for a rather large streaked area, most of the protein is impacted against the forward edge of the sample slot. This behavior indicates that the bulk of the protein in this system exists in the form of high molecular weight complexes, of a size sufficient to prevent their passage through the gel pores. κ-Casein exhibits an identical electrophoretic behavior when run in gels composed of a dissociating system such as tris-8 *M* urea; this indicates that other bonds in addition to noncovalent ones are responsible for the high molecular weight of κ-casein.

Reduction of the disulfide bonds of κ-casein

[2] Reference to particular products or companies does not imply their endorsement by the U. S. Department of Agriculture over others not mentioned.

[3] Cyanogum 41 gelling agent, a mixture of acrylamide and *N*,*N*[1]-methylenebisacrylamide, was obtained from Fischer Scientific Company, Fairlawn, New Jersey.

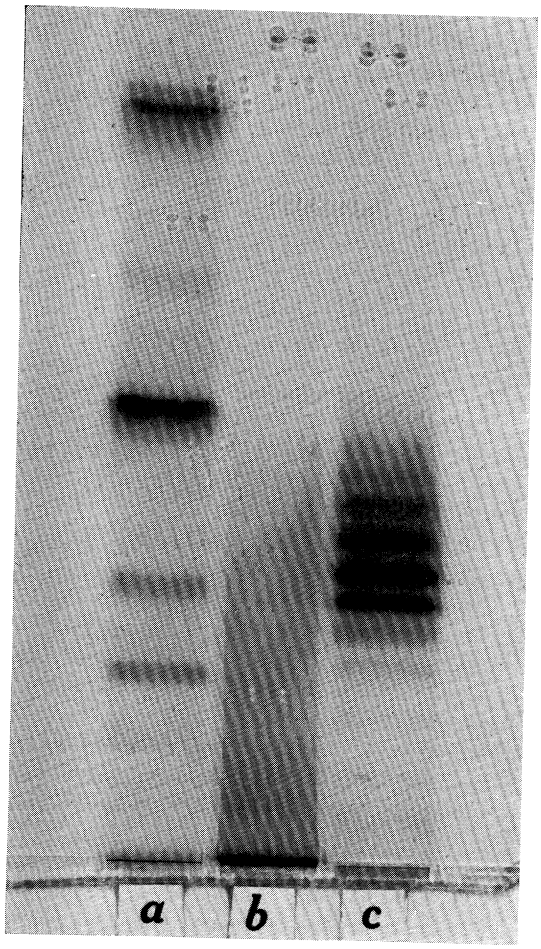

FIG. 1. (*a*) Polyacrylamide-gel electrophoresis of whole casein; (*b*) "native" κ-casein; and (*c*) reduced nonalkylated κ-casein.

with 2-mercaptoethanol converted the protein into a size which readily migrated through the gel and permitted resolution of reduced κ-casein into individual electrophoretic components. The electrophoretic pattern of reduced nonalkylated κ-casein obtained from pooled milk is shown in Fig. 1c. Three major components were readily detected together with five or six minor components; no trace of protein was observed in the sample slot.

The three major electrophoretic components of pooled κ-casein are identical with the A, B, and C bands observed to occur in various combinations in reduced κ-caseins obtained from the milks of individual cows (6). These components appear to be variant forms; however, genetic control for the observed polymorphism of κ-casein has not yet been established. The minor components seen in the electrophoretic patterns may also be variant forms or they may be due to modifications of the major components through loss of amide groups of sialic acid moieties. Identical electrophoretic patterns have been obtained for reduced κ-casein after reduction of whole, acid-precipitated casein and after reduction of κ-casein isolated by the urea-H_2SO_4 procedure (7). It is doubtful that such alterations could occur as a result of the isoelectric precipitation; however, incomplete biosynthesis may also be involved in the formation of the minor κ-casein components.

On the basis of the electrophoretic patterns in Fig. 1, it can be concluded that a number of polypeptide components are linked together through disulfide bonds in κ-casein preparations. This evidence, together with the lower molecular weights obtained for κ-casein by sedimentation analysis after disulfide cleavage (3), strongly suggests that extensive intermolecular disulfide bonding, possibly occurring in a random manner, is responsible in part for the formation of high molecular weight κ-casein.

The above observations, however, do not exclude the existence of intramolecular disulfide bonds in κ-casein, nor do they permit the assumption that the individual electrophoretic components observed after reduction represent different κ-casein molecules.

Reduction of the disulfide bonds of κ-casein, as obtained in Fig. 1, made it impossible to determine whether "native" κ-casein molecules are composed of one or more polypeptide chains. In an attempt to obtain partially reduced κ-casein, the effect of reduction with varied ratios of mercaptoethanol to half-cystine content (1:10 to 10:1) and for varied periods of time (5 minutes to 24 hours) was investigated. In all cases the patterns were identical with that of Fig. 1c, with the exception that some unreduced protein still remained in the sample slot at low ratios of reductant and after short reduction times. Similar results were obtained when reduction was done with

urea concentrations of 0–8 *M*. The failure to observe partially reduced forms of κ-casein which might have indicated the sequence of cleavage indicated that all of the disulfide bonds are probably equally accessible. More specific techniques capable of distinguishing between intramolecular and intermolecular disulfide bonds will be required before the nature of disulfide bonding in κ-casein can be elucidated.

The need for stable reduced forms of κ-casein prompted the preparation of the CNE, CM, and CAM-κ-casein derivatives. The electrophoretic patterns of these derivatives were obtained and compared with that of the reduced nonalkylated κ-casein. In all cases the patterns of the derivatives were qualitatively identical with the nonalkylated protein. In the case of the CM-κ-casein, the addition of charged groups (—COOH) caused an increase in the electrophoretic mobilities, whereas with the iodoacetamide derivative, CAM-κ-casein, the mobilities were somewhat slower probably due to nonspecific alkylation of methionine residues (15). The electrophoretic mobilities of components in CNE-κ-casein were identical with those of the reduced nonalkylated κ-casein.

No residual sulfhydryl groups were found in CNE-κ-casein by the spectrophotometric ferricyanide procedure (10). Similarly, the absence of even a trace of cysteine in the amino acid elution pattern (11) of CNE-κ-casein established that complete reduction and alkylation of all disulfide bonds in κ-casein achieved. *S*-Carboxyethylcysteine, the hydrolysis product of *S*-cyanoethylcystein, was well resolved and emerged between serine and glutamic acid.

Stabilization of α_s-casein and rennin sensitivity. The marked effect of disulfide cleavage in decreasing the molecular weight of κ-casein (3) and on its electrophoretic behavior raised the question of whether reduced κ-casein retained the characteristic properties of α_s-casein stabilization and rennin sensitivity. These properties have been useful in investigating the effects of various treatments on κ-casein.

The ability of κ-casein to stabilize the calcium-sensitive casein (α_s) has been measured by the determination of the amount of α_s-casein solubilized in the presence of 0.02 *M* calcium chloride with various ratios of κ to α_s-casein (12). All three reduced derivatives quantitatively retained in α_s-casein stabilizing ability

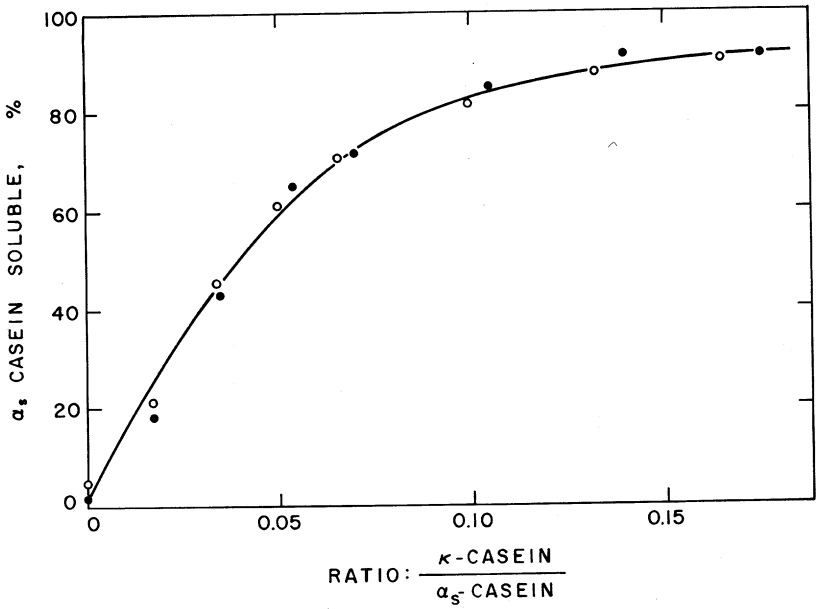

FIG. 2. Solubilization of α_s-casein by "native" (—●—) and CNE-κ-casein (—○—).

equal to the nonreduced κ-casein. Only the results of unreduced and CNE-κ-caseins are shown in Fig. 2, wherein the curves indicate approximately 90% solubilization at κ/α_s ratios of 0.15.

It would appear that all of the stabilizing groups in κ-casein are accessible and available for κ/α_s complex formation, otherwise a greater stabilization might have been observed at a given CNE-κ/α_s ratio if additional stabilizing groups were exposed as a result of reduction. It is also possible, however, that since both κ-casein and reduced κ-casein undergo considerable aggregation, some stabilizing groups may still remain unaccessible.

The action of rennin on CNE-κ-casein was followed by the determination of nitrogen and sialic acid soluble in 12% TCA released during digestion. The results obtained after the incubation of a 0.5% of CNE-κ-casein with rennin (1.0 μg per milliliter) at pH 6.7 are shown in Fig. 3. Turbidity developed within 5 minutes, and a heavy precipitate of *para*-CNE-κ-casein was evident after 10 minutes. Approximately 13% of the total nitrogen and 90% of the total sialic acid were found in the 12% TCA fraction after 20 minutes digestion. The values are in agreement with those obtained with unreduced κ-casein.

The quantitative retention of the stabilizing ability and rennin sensitivity by the reduced derivatives of κ-casein is quite interesting. The stabilization results obtained with the CNE-κ-casein permit several conclusions regarding the nature of the complex formation between α_s- and κ-casein. Obviously, neither intact disulfide bonds nor free sulfhydryl groups are required. This indicates that if the κ-casein molecule consists of two or more polypeptide chains bound through disulfide linkages, this molecular integrity is not required for stabilization to occur. From a functional standpoint it appears that intact individual polypeptide chains are all that are required. The question whether native κ-casein molecules consist of more than one polypeptide chain cannot be resolved from this study. However, the inability to obtain partially reduced forms might be considered as evidence for a single polypeptide chain structure for κ-casein.

It does not appear that α_s-casein stabilization and complex formation are obtained from the combined effect of several of the different electrophoretic components. Preliminary studies of individual components obtained by chromatographic fractionation indicate that the characteristic κ-casein properties also reside within the individual polypeptide chains.

The evidence obtained from reduced

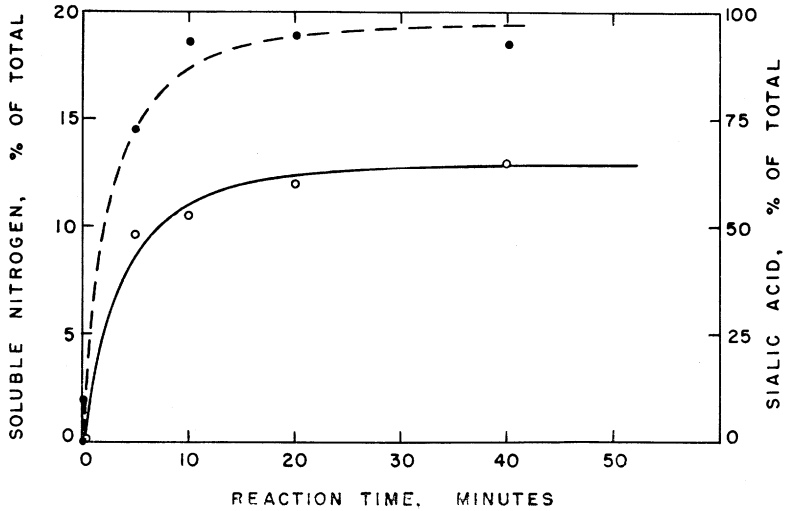

FIG. 3. Release of 12% TCA-soluble nitrogen (—○—) and sialic acid (- -●- -) from CNE-κ-casein by the action of rennin.

κ-casein thus far suggests that κ-casein preparations as isolated from whole casein consist of a spectrum of molecular weights which arise partly as a result of intermolecular disulfide bonding between a number of κ-casein molecules. These oxidations between free sulfhydryl groups (16) of individual κ-casein molecules could occur either before or after milking. Cleavage of these intermolecular disulfide bonds and the presence of a deaggregating solvent results in a lowered molecular weight of approximately 20,000 (3) for the basic polypeptide unit.

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REFERENCES

1. WAKE, R. G., AND BALDWIN, R. L., *Biochim. Biophys. Acta* **47,** 225 (1961).
2. WOYCHIK, J. H., *Federation Proc.* **23,** (Pt. 1), 474 (1964).
3. SWAISGOOD, H. E., AND BRUNNER, J. R., *Biochem. Biophys. Res. Commun.* **12,** 148 (1963).
4. SWAISGOOD, H. E., AND BRUNNER, J. R., *J. Dairy Sci.* **45,** 1 (1962).
5. NEELIN, J. M., *J. Dairy Sci.* **47,** 506 (1964).
6. WOYCHIK, J. H., *Biochem. Biophys. Res. Commun.* **16,** 267 (1964).
7. ZITTLE, C. A., AND CUSTER, J. H., *J. Dairy Sci.* **46,** 1183 (1963).
8. MCKENZIE, H. A., AND WAKE, R. G., *Biochim. Biophys. Acta* **47,** 240 (1961).
9. WEIL, L., AND SEIBLES, T. S., *Arch. Biochem. Biophys.* **95,** 470 (1961).
10. KATYAL, J. M., AND GORIN, G., *Arch. Biochem. Biophys.* **82,** 319 (1959).
11. PIEZ, K. A., AND MORRIS, L., *Anal. Biochem.* **1,** 187 (1960).
12. ZITTLE, C. A., *J. Dairy Sci.* **44,** 2101 (1961).
13. WARREN, L., *J. Biol. Chem.* **234,** 1971 (1959).
14. PETERSON, R. F., *J. Dairy Sci.* **46,** 1136 (1963).
15. GUNDLACH, H. G., STEIN, W. H., AND MOORE, S., *J. Biol. Chem.* **234,** 1754 (1959).
16. BEEBY, R., *Biochim. Biophys. Acta* **82,** 418 (1964).